

## Antibodies against the extracellular enveloped virus B5R protein are mainly responsible for the EEV neutralizing capacity of vaccinia immune globulin

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### Abstract

In the event of smallpox bioterrorism, widespread vaccination may be required. Vaccinia immune globulin (VIG) has been used to treat complications from the smallpox vaccine. While the potency of VIG was defined by its ability to neutralize intracellular mature virus, a second form of vaccinia called the extracellular enveloped virus (EEV) is critical for virus spread in the host. The B5R-protein is one of many EEV-specific proteins. Immunoprecipitation and ELISA revealed that VIG recognizes the B5R-protein. An EEV plaque-reduction assay using a recombinant vaccinia that lacks the majority of the extracellular domain of B5R showed that the ability of VIG to neutralize EEV is principally directed at B5R. In addition, absorbing out the anti-B5R antibody present in VIG through the addition of recombinant B5R protein abrogated VIG's ability to significantly neutralize wild-type EEV. This work demonstrates the prominent role of B5R as a target of EEV-neutralizing activity of human antibodies.

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### Introduction

Concerns about the intentional release of variola virus has led to the possibility of widespread vaccination with vaccinia virus. The primary treatment for vaccinia vaccine complications would be vaccinia immune globulin (VIG). While never tested in randomized clinical trials, VIG is believed to be effective in treating complications resulting from vaccinia virus vaccination (Barbero et al., 1955; Kempe et al., 1956; Sussman, 1965). VIG is prepared by cold ethanol fractionation of plasma obtained from multiple donors who were recently vaccinated with vaccinia virus. In prior decades, the donors may have been previously vaccinated as children, and then revaccinated a few weeks before provid-

ing plasma for VIG preparation. Given the fact that routine vaccinia virus vaccination ended in the United States in the early 1970s, current VIG stocks are being prepared from donors who are vaccinated once, approximately 2 weeks before plasmaphoresis. The potency of VIG was defined by its ability to neutralize intracellular mature virus (IMV). However, a second form of vaccinia virus known as the extracellular enveloped virus (EEV) is critical for virus spread in the host (Payne, 1980; Smith and Vanderplasschen, 1998). The B5R protein is one of many EEV-specific proteins that are vital for the optimal formation of EEV (Engelstad and Smith, 1993; Engelstad et al., 1992; Isaacs et al., 1992; Wolffe et al., 1993). While it has been shown that the B5R protein is an important target of EEV neutralizing antibodies (Galmiche et al., 1999; Hooper et al., 2003; Law and Smith, 2001), here we show that human immune globulin used as a therapeutic agent to treat complications from vaccinia virus vaccination contains a high titer of antibodies that recognize B5R and, using a recombinant

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vaccinia virus that lacks the majority of the extracellular domain of the B5R protein, we demonstrate that the ability of VIG to neutralize EEV is mainly directed against B5R. This finding more clearly defines the role of B5R as a critical target of EEV neutralizing antibody than has been suggested by previous work (Law and Smith, 2001) using hyperimmune vaccinia globulin obtained from a vaccinated rabbit.

## Results

### VIG recognizes B5R by immunoprecipitation and ELISA

Using a previously constructed recombinant vaccinia virus that expresses a secreted soluble B5R protein (Isaacs et al., 1992), we demonstrated that VIG recognized B5R secreted into the media of infected cells by immunoprecipitation (Fig. 1A). Direct ELISA demonstrated high-titer anti-B5R antibodies present in VIG (Fig. 1B). Strong reactivity (O.D.  $\leq 1$ ) with recombinant vaccinia virus produced soluble-secreted B5R, bacterial-produced B5R, and baculovirus-produced B5R was obtained with VIG at a dilution of 1:25 600 (0.39  $\mu\text{g}$ ; Fig. 1B). In contrast, control Ig had reactivity with the bound proteins at only a 1:800 dilution (12.5  $\mu\text{g}$ ). The low reactivity of control pooled human immune globulin with vaccinia virus proteins on immunoprecipitation and ELISA is likely due the fact that this pooled immune globulin likely contains immune globulin from some donors who had been previously vaccinated with vaccinia virus (Goldsmith et al., 2004).

### VIG neutralizes EEV mainly by targeting B5R

The outer membrane of EEV is considered to be quite fragile and thus harsh manipulations like freeze-thawing or even prolonged storage at 4 °C results in release of the equally infectious IMV. Therefore, assays to examine EEV

neutralization were carried out with fresh EEV incubated along with a potent IMV-neutralizing antibody 2D5 (Ichihashi et al., 1994) to neutralize any contaminating IMV. VIG at 1.5 mg/ml results in  $\sim 81\%$  neutralization of wild-type EEV (vaccinia virus strain IHDJ), while the same concentration of control human Ig has no effect (Fig. 2A). This sensitivity of wild-type EEV to neutralization by VIG is in contrast to a mutant vaccinia virus that lacks approximately 80% of the extracellular domain of the B5R protein, which is resistant to neutralization by VIG (Fig. 2B). As further evidence that the ability of VIG to neutralize EEV is largely due to B5R, we competed out anti-B5R antibodies from VIG by incubating VIG with recombinant B5R protein before adding VIG to the EEV neutralization assay. When recombinant baculovirus-produced B5R protein (Earl et al., 2004) is included (100  $\mu\text{g}/\text{ml}$ ), VIG was only able to neutralize approximately 20% of wild-type EEV (Fig. 2C). Importantly, this loss of EEV neutralizing activity is not caused by a nonspecific alteration of the VIG due to the addition of a baculovirus-produced protein. When we added an irrelevant baculovirus-produced protein (herpes simplex virus extracellular domain of gD; Rux et al., 1998) to VIG, it did not alter VIG's ability to neutralize EEV (Fig. 2C). Because a previously published study (Law and Smith, 2001) using a WR-based recombinant vaccinia virus lacking approximately 80% of the ectodomain of the B5R protein (Herrera et al., 1998) and hyperimmune rabbit immune globulin gave a different result, we also examined EEV neutralization of this WR-based virus by VIG (Fig. 3D). Similar to the IHDJ-based virus lacking all four SCR domains (Fig. 3B), the WR-based virus was also relatively resistant to neutralization by human VIG, indicating that our findings were not vaccinia-strain specific. In addition, to ensure that our findings using VIG (Baxter) obtained from the CDC were not brand- or lot-specific, we found similar EEV neutralization activity with a different source of VIG (VIG-IV, Reference vaccinia immune globulin) obtained from the FDA (Fig. 3).

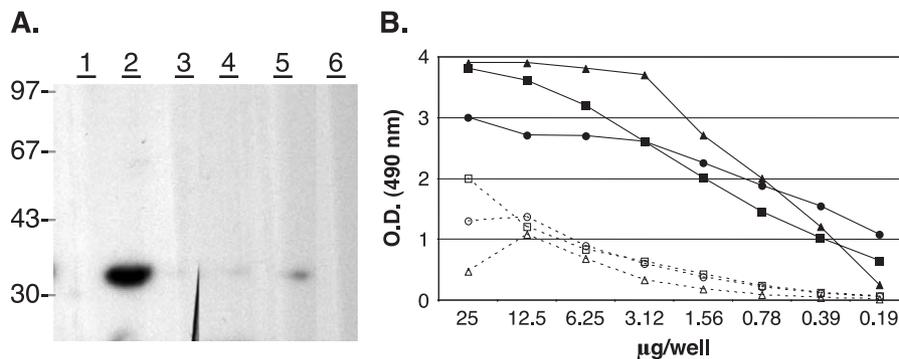


Fig. 1. Reactivity of VIG with B5R. (A) Immunoprecipitations were performed with media from cells infected with wild-type (WT) vaccinia virus that does not contain B5R or with media from a recombinant vaccinia virus that secretes a soluble form of B5R (sB5R). Successful immunoprecipitation of B5R was detected using an anti-B5R rabbit antibody (Isaacs et al., 1992). Lane 1, VIG with WT media; lane 2, VIG with sB5R; lane 3, beads with WT media; lane 4, control-Ig with WT media; lane 5, control-Ig with sB5R; lane 6, beads with sB5R. Numbers on the left refer to the molecular masses (in kilodaltons). (B) Direct ELISA of dilutions of VIG (closed symbols, solid lines) and control Ig (open symbols, broken lines) with sB5R (triangles), baculovirus-produced B5R (squares), and bacteria-produced B5R (circles).

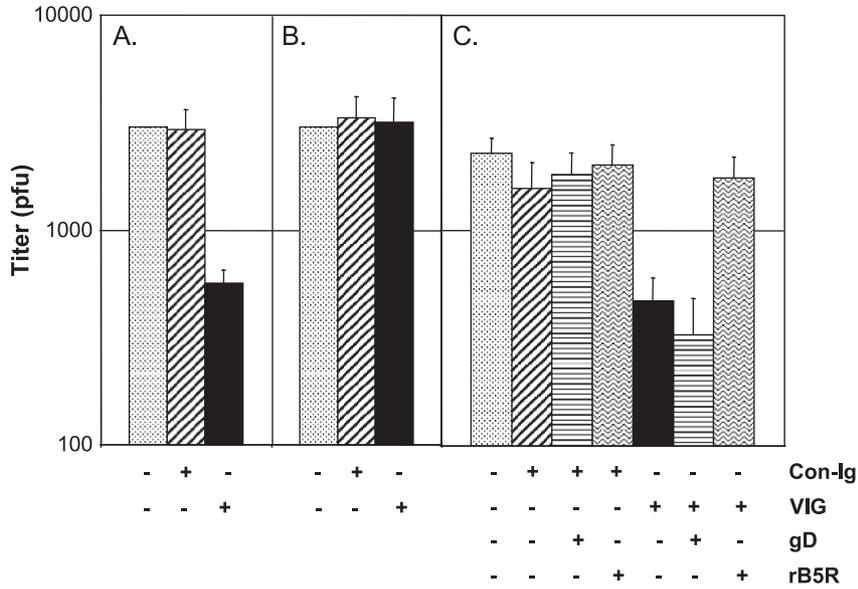


Fig. 2. EEV neutralization by VIG. EEV from cells infected with wild-type virus (A) or from the mutant virus lacking the majority of the extracellular domain of B5R (B) were incubated in the presence of a potent IMV-neutralizing monoclonal antibody along with no antibodies (gray bars), control pooled Ig (Con-Ig; hatched bars), or VIG (black bars) and the resulting titer was determined by serial dilutions. Data was normalized to an input virus of 3000 pfu and represents the mean of five independent experiments for each condition. Wilcoxon rank sum test used for comparison between each control Ig and VIG condition, (A)  $P = 0.009$  and (B)  $P > 0.5$ , both two-tailed. When recombinant-soluble rB5R baculovirus-produced protein (rB5R; 10  $\mu\text{g}/\text{sample}$ ) was included during incubation (C), the ability of VIG to neutralize wild-type EEV was significantly reduced, while addition of a control baculovirus-produced protein (gD) did not affect VIG's ability to neutralize EEV. + and - refer to the addition or absence of VIG or control Ig (Con-Ig) or the addition or absence of soluble gD or rB5R. Data represents the mean of three independent experiments for each condition. Student's  $t$  test used for comparison between VIG alone and VIG with gD ( $P = 0.29$ ) or VIG with rB5R ( $P = 0.009$ ), both two-tailed.

*VIG inhibits comet formation independent of B5R*

While direct neutralization of released virus is one way, antibodies can help control orthopoxvirus infections; inhibiting the release of virus from infected cells is another

potential mechanism. When grown in a cell monolayer under liquid media, vaccinia virus (strain IHDJ) generates a characteristic plaque phenotype called comets (Fig. 4A) due to the release of EEV from infected cells and spread to distant cells (Apleyard et al., 1971; Law et al., 2002).

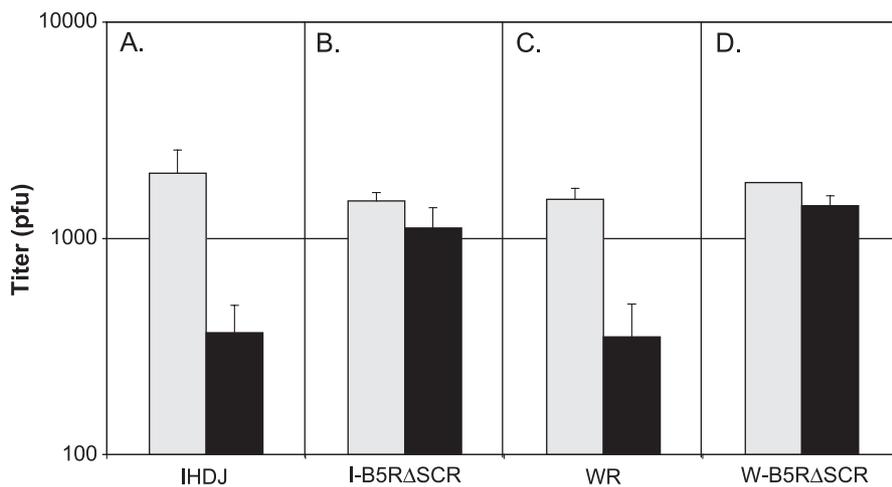


Fig. 3. Comparison of neutralization of EEV from strains IHDJ and WR by VIG. EEV from cells infected with wild-type virus, strain IHDJ (A), or from the mutant virus lacking the majority of the extracellular domain of B5R, strain IHDJ (B), or from cells infected with wild-type virus, strain WR (C), or from the mutant virus lacking the majority of the extracellular domain of B5R, strain WR, were incubated in the presence of a potent IMV-neutralizing monoclonal antibody along with no antibodies (gray bars), or FDA Reference VIG-IV (black bars) and the resulting titer was determined by serial dilutions. Data represents the mean of three to four independent experiments for each condition. Student's  $t$  test used for comparison between no antibody and VIG-IV in each panel (A)  $P = 0.003$ , (B)  $P = 0.15$ , (C)  $P = 0.004$ , and (D)  $P = 0.59$ , all two-tailed.

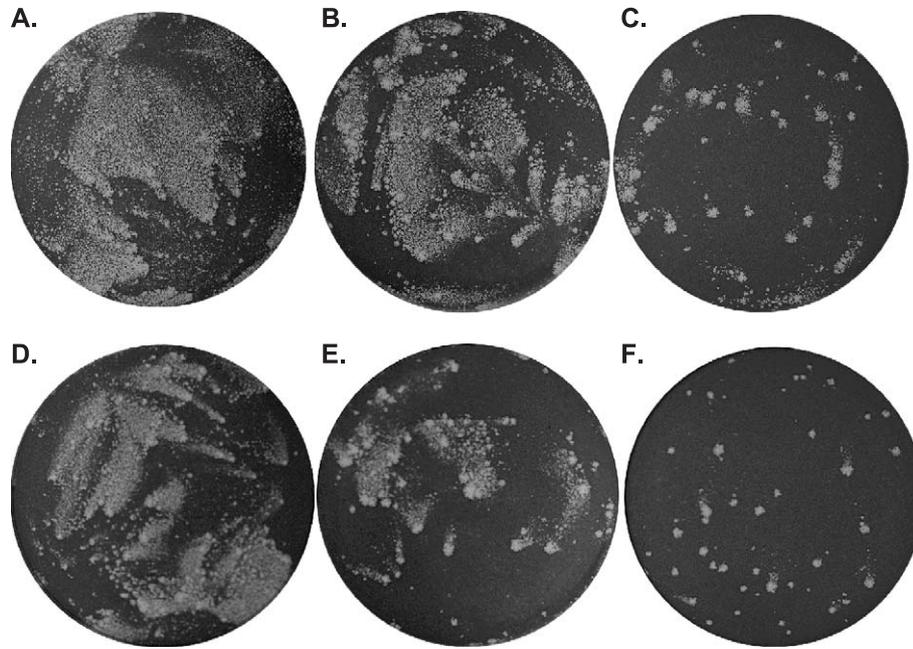


Fig. 4. Plaque phenotypes in the presence and absence of antibodies. Vaccinia virus (strain IHDJ) generates a characteristic comet phenotype when grown in monolayers overlaid with liquid media (A, D) and in the presence of control Ig (250 µg/ml; B, E). However, comet formation is appreciably inhibited in the presence of VIG (250 µg/ml; C, F). Wild-type vaccinia virus (A, B, C); I-B5RΔSCR<sub>1-4</sub> (D, E, F).

Similar comets are generated by the recombinant vaccinia virus lacking the majority of the extracellular domain of the B5R protein (Herrera et al., 1998) (Fig. 4D). In the presence of control Ig, there is a slight alteration in comets, but comets are still formed (Fig. 4B,E). However, the comet phenotype is appreciably inhibited in the presence of VIG in cells infected with either the wild-type (Fig. 4C) or mutant virus lacking the extracellular domain of the B5R protein (Fig. 4F). This indicates that VIG has targets that inhibit comet formation that are independent of B5R protein.

## Discussion

Recent data (Hammarlund et al., 2003) suggests that vaccination against smallpox with vaccinia virus leads to long-lasting immunity that protects from death. These data also highlight the apparent importance of antibody responses as a correlate of immunity. Therefore, a more thorough understanding of VIG, a product successfully used to treat complications from vaccinia virus vaccination and for prophylaxis after smallpox exposure (Barbero et al., 1955; Kempe et al., 1956; Sussman, 1965), is warranted. One would expect that important antibody responses would be directed at the surface proteins found on the two main forms of infectious virus, IMV and EEV. IMV enters the cell in a pH-independent fusion at the cell membrane and VIG is quite potent at neutralizing IMV. EEV is thought to gain entry into the cell through an endocytic pathway that requires acidification (Vanderplasschen et al., 1998a). This may explain why EEV is more difficult to neutralize. Once

in the acidic environment of the endosome, the fragile outer membrane of EEV is disrupted, releasing the equally infectious IMV, which can then enter the cell in an environment where IMV-neutralizing antibodies are no longer effective. Thus, understanding the targets of EEV-neutralizing antibodies will be important for the development of more effective antibody therapies directed at poxvirus infections as well as the development of safer smallpox vaccines.

The B5R protein is an important target of EEV neutralizing antibodies (Galmiche et al., 1999; Hooper et al., 2003; Law and Smith, 2001). B5R is a type I membrane protein with a large extracellular domain composed of regions with similarity to short consensus repeat (SCR) domains of complement regulatory proteins (Engelstad et al., 1992). While no complement regulatory activity has been attributed to this protein (Vanderplasschen et al., 1998b), the B5R protein is involved in the wrapping steps of IMV that ultimately form EEV. The SCR domains of the B5R protein are not required for EEV formation (Herrera et al., 1998; Mathew et al., 1998) and we used a recombinant vaccinia virus lacking the four SCR domains to characterize VIG's ability to neutralize EEV. Our results showing that this mutant virus lacking the SCR domains is resistant to neutralization by vaccinia-immune polyclonal human IgG differs from a report using vaccinia-immune polyclonal rabbit IgG (Law and Smith, 2001). The authors of that paper concluded that EEV neutralization by hyperimmune globulin had multiple targets and the lack of any one EEV-specific protein did not result in the ability of virus to completely escape neutralization. Furthermore, they showed that the recombinant vaccinia virus lacking

all four SCR domains was fully sensitive to neutralization with hyperimmune rabbit globulin while we show that this virus is resistant to neutralization by human VIG. The reason for the discrepancy is unclear. A likely explanation is that their work was with hyperimmune IgG obtained from a single rabbit boosted multiple times over a 3-month period with  $2 \times 10^7$  pfu of vaccinia virus (strain WR), whereas we studied VIG, pooled sera that had been collected from many humans recently vaccinated once with Dryvax. Thus, there may be a difference in the quality and targets of the antibody response developed in rabbits vs. humans under these different vaccination schedules. Although Law and Smith used a WR-based recombinant vaccinia virus lacking the B5R extracellular domain that we generated (Herrera et al., 1998), this virus when incubated with VIG performs similarly to the IHDJ-based virus that we focused on in this report. Similar to Law & Smith, we used the same cell line to generate EEV. Other potential explanations for the differences in the two studies include that our assays looked to neutralize 20 times more EEV than they used in their assays (i.e., approximately 3000 input pfu in our assay vs. 150 pfu in their assay). Consequently, we used a higher concentration of immune globulin in our assays (i.e., 1500  $\mu\text{g/ml}$  in our assay vs. 100  $\mu\text{g/ml}$  in their assay). So perhaps our assay is more sensitive to neutralization via cross-linking and aggregation of virus. In support of the central role of B5R as a target of EEV neutralizing antibody that we report here, Law and Smith show that antibodies raised to B5R could fully neutralize wild-type EEV to a level similar to hyperimmune vaccinia virus IgG (Law and Smith, 2001). Thus, we believe that this data supports our conclusion that antibodies directed at B5R that form during vaccination are mainly responsible for EEV neutralization. Because EEV is important for orthopoxvirus pathogenesis, the B5R protein will likely be a key therapeutic target for future antibody therapies or new subunit or DNA vaccines directed against smallpox (Galmiche et al., 1999; Hooper et al., 2003, 2004).

While the ability of VIG to neutralize EEV is directed at B5R, using the comet inhibition assay as an *in vitro* measure of the release of EEV from infected cells, we found that VIG could inhibit comet formation independent of the presence or absence of the majority of the extracellular domain of the B5R protein. Thus, the humoral immune response generated by vaccinia virus vaccination has additional targets that can limit the spread of infection. That is, while some antibodies may not be able to neutralize free EEV, antibodies to EEV-specific proteins may prevent release of EEV from infected cells. This may be a mechanism by which antibodies raised against the EEV-specific proteins that fail to neutralize EEV confer protection in animal models (Galmiche et al., 1999). It is unknown whether EEV neutralizing activity or comet inhibition activity is more important for protection, but some data (Galmiche et al., 1999) suggest that either activity can be equally protective.

## Materials and methods

### Viruses

Freshly prepared EEV was obtained from serum-free media (Opti-MEM) of RK-13 cells infected for 48 h with vaccinia virus [International Health Department Strain-J (IHDJ)] or with a previously described recombinant vaccinia virus vSI-26 (I-B5R $\Delta$ SCR<sub>1–4</sub>) (Herrera et al., 1998). I-B5R $\Delta$ SCR<sub>1–4</sub> is an IHDJ-based recombinant virus that lacks 206 residues of its 257 amino acid B5R extracellular domain, but produces near wild-type levels of EEV (Herrera et al., 1998). The harvested media containing EEV was kept at 4 °C and used within 1–2 weeks after initial infection. Titers of EEV in such media (in the presence of anti-IMV neutralizing antibody) were routinely approximately  $10^6$  plaque-forming units (pfu)/ml. In a similar fashion, EEV from vaccinia virus Western Reserve (WR) and vSI-22, a previously described WR-based mutant virus lacking the B5R-ectodomain (W-B5R $\Delta$ SCR<sub>1–4</sub>) (Herrera et al., 1998), were prepared from RK-13 cells.

### Antibodies

The anti-IMV neutralizing monoclonal antibody, 2D5 (Ichihashi et al., 1994), directed at the vaccinia virus L1R protein (Wolffe et al., 1995), was used at a 1:1000 dilution, a dilution that is able to neutralize 4–5 log<sub>10</sub> of IMV. Two sources of Vaccinia immune globulin (VIG) were used. VIG (Baxter; 165 mg/ml; Lot No. 0448A101AA) was obtained from the CDC and VIG-IV (Reference vaccinia immune globulin; 50 mg/ml; Lot 1) was obtained from the FDA. Control pooled human immune globulin (Ig) used was Polygam (Red Cross; 100 mg/ml).

### Immunoprecipitations

Serum-free media from cells infected with wild-type vaccinia virus or a previously described virus, vSI-13 (Isaacs et al., 1992), were used in immunoprecipitations. vSI-13 is a recombinant vaccinia virus that expresses a mutated version of the B5R open reading frame with a stop codon just before the transmembrane domain that results in the secretion of the extracellular domain of the B5R protein into the media. Two microliters of this media or 10  $\mu\text{l}$  of media from cells infected with wild-type virus (that does not secrete B5R) were incubated with 250  $\mu\text{g/ml}$  of VIG or control Ig at 4 °C overnight. Protein G beads were then added and incubated for 2 h at room temperature. After washing, the beads were boiled in Laemmli loading buffer and run on a 0.1% SDS–16% PAGE gel. Proteins were transferred to PVDF membrane. The membrane was blocked with 10% nonfat milk in PBS for 2 h at room temperature, washed three times with PBS containing 0.1% Tween 20, and then incubated with a previously described (Isaacs et al., 1992) anti-B5R rabbit antibody (1:5000). After three washes, the blot was incubat-

ed with HRP-conjugated goat antirabbit antibody (1:5000) for 1 h and immunoreactive bands were visualized by chemiluminescence.

#### Enzyme-linked immunosorbent assay (ELISA)

A direct anti-B5R ELISA was carried out in parallel on recombinant B5R from three sources: the vaccinia virus expressed soluble B5R protein (sB5R) (Isaacs et al., 1992) described earlier, a GST-fusion protein expressed in *Escherichia coli* (bact-B5R; Shamim and Isaacs, unpublished), and a His-tagged B5R protein expressed in baculovirus (baculo-B5R; (Earl et al., 2004)). Ninety-six-well microtiter ELISA plates were coated with 5 µl/ml of sB5R or 10 µg/ml of either bact-B5R or baculo-B5R in coating buffer, incubated overnight at 4 °C, and then blocked with 2% nonfat milk in PBS for 2 h. Serial dilutions of VIG and control human Ig (both at 100 mg/ml and starting at 1:400 dilution and out to 1:51,200) were added to wells, incubated for 1 h at 37 °C, and washed with PBS containing 0.05% Tween 20. Bound antibody was detected with HRP-conjugated goat antihuman antibody (1:10,000) and chromogen orthophenylenediamine (1 mg/ml) in 50 mM citrate buffer (pH 5.0) at 490 nm.

#### EEV neutralization assay

The assay was carried out in a final volume of 100 µl with an input of approximately 3000 pfu of EEV in serum-free media along with anti-IMV neutralizing monoclonal antibody 2D5 (1:1000 final concentration) with or without 150 µg of immune globulins (VIG or control Ig). Samples were incubated for 1–2 h at 37 °C and then infectious virus was titered by serial dilutions. Dilutions were added to monolayers of BSC-1 cells and incubated for 2 h at 37 °C in a 5% CO<sub>2</sub> atmosphere. The inoculum was removed, the wells overlaid with media containing 2.5% heat-inactivated FBS and 1% carboxymethylcellulose, and plates were incubated for approximately 2 days. The overlay was removed, the cells stained with 0.1% crystal violet, and the plaques were counted.

#### Comet inhibition assay

Confluent BSC-1 cells were infected with vaccinia virus (strain IHDJ) and approximately 2 h postinfection, the inoculum was removed and replenished with fresh serum-free media containing 250 µg/ml of VIG or control Ig. Plates were incubated for 36 h and then stained with crystal violet. This assay was performed on multiple occasions and representative wells were photographed.

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